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Cyclic Peroxide Acids and a new fatty acid from Okinawan Sponge *Plakortis* sp.

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Abstract

Bioassay-guided fractionation of a cytotoxic extract of an Okinawan sponge *Plakortis* sp. resulted in the isolation of a known cyclic peroxide methyl ester (1a) along with two new cyclic peroxide acids (1b and 2b) and a new aliphatic fatty acid (3). Compound 2b was confirmed to be an isomer of 1b by NMR and MS. Moreover, careful comparison of the spectroscopic data of 2b with that of peroxyplakoric acid A₁ methyl ester (2a) suggested that compound 2b was identical with 2a. Treatment of 2b with TMS diazomethane yielded a compound identical with 2a with respect to NMR and MS. This data confirmed that the cyclic peroxidases 1b and 2b occurred naturally.

Additionally, the structure of 3 was confirmed by 1D and 2D NMR experiments. A plausible biosynthetic pathway has been proposed that rationalizes transformations in the ring-opening of the cyclic peroxide. Compound 1a was evaluated to have IC₅₀ 8.7 µg/mL against NBT-T2 cells while compound 3 was inactive. This result suggested that the cyclic peroxide moiety could be responsible for the cytotoxicity of the sponge constituents.

Keywords: *Plakortis* sp., cyclic peroxide, fatty acid, 1D and 2D NMR, cytotoxicity.

Introduction

The sponge of the genus *Plakortis* is well known as a prolific source of biologically active secondary metabolites.¹ Cyclic peroxides with different side chains are frequently isolated from this genus of sponge and have been reported to have antifungal⁹, antimalarial², antimicrobial¹³ and antitumor⁵ activity. The sponge is well known as a source of peroxy ring-containing compounds plakortin¹⁰ along with the cyclic peroxide containing the unsaturated side chain and methyl ester form⁶. In search of new biologically active molecules from marine organisms, we have isolated three cyclic peroxides (1a, 1b and 2b) and a fatty acid (3) from a marine sponge *Plakortis* sp. collected off Iribu Island, Okinawa. In particular, compounds 1a, 1b and 2b contained the identical side chain, but the substituents and configurations on a 1,2-dioxane ring were dissimilar between the three compounds.

Herein, we describe the isolation and stereo structural characterization of the isolated compounds together with

their biological activities. The antifungal structure-activity relationships of the cyclic peroxides (1–3) and the rearrangement product (1b) were evaluated for its cytotoxicity against rat bladder tumor cells NBT-T2.

Materials and Methods

General: ¹H, ¹³C and 2D NMR (COSY, HSQC and HMBC) spectra were obtained on a Bruker Avance III 500 NMR spectrometer in CDCl₃ with reference to an internal standard of TMS. Chemical shifts and coupling constants were given as δ and Hz. FTIR spectrum was taken on a Varian FTS-3000 instrument. HRESIMS measurements were performed on a Jeol JMS-T 100LP mass spectrometer and the molecular formulas were analyzed using a program Mass Center (Version 1.3.10, MS_56010 MP). Purification was done with Hitachi or Shimadzu HPLC instruments using Cosmosil (Nacalai) or Mightysil (Wako) columns.

Animal materials: The sponge *Plakortis* sp. was collected off Iribu Island in Okinawa by using SCUBA diving and kept frozen until extraction with code MJ-JT-12-560.

Extraction and Isolation: The collected specimen was extracted with acetone (500 mL) thrice. After solvent removal using rotary evaporation, the extract was partitioned between EtOAc and water. The EtOAc layer was concentrated to give the lipophilic fraction and the aqueous layer was dissolved in MeOH to give the more polar fraction. The fractions were tested for their cytotoxicity against NBT-T2 cells (at 10 and 100 µg/mL). The active EtOAc extract (372.5 mg) was separated using silica gel column chromatography with eluents: *n*-hexane:EtOAc (4:1, 3:1, 2:1), CH₂Cl₂, EtOAc and EtOAc:MeOH (1:1) to give six fractions.

The second fraction (16.9 mg) was further separated by using normal phase HPLC with *n*-hexane: EtOAc mixture as an eluent furnished 13 fractions. The fifth fraction (9.3 mg) was purified to give compound 1a (2.4 mg) by using reversed phase HPLC with MeOH: H₂O (2:3) as a solvent system. The EtOAc fraction (18.1 mg) was subjected to normal phase HPLC using *n*-hexane: EtOAc (1:1) as the solvent system, this yielded compounds 1b and 2b.

The CH₂Cl₂ (15.8 mg) fraction was subjected to normal phase HPLC resulting in five fractions. The third fraction from this was separated by using PTLC with once elution to give compound 3 (1.1 mg).

Methylation of compound 2b: Excess TMSCHN₂ was added to a solution of compound 2b (0.1 mg) dissolved in MeOH (100 μ L). The reaction was monitored by TLC. After an hour, the solution was concentrated under nitrogen flow.

Cytotoxic Assay: NBT-T2 cells derived from chemically induced rat bladder carcinoma cells were used in screening and evaluating the cytotoxic activity⁷. NBT-T2 cells (Riken Bio Resource Center) cultured in Dulbecco's Modified Eagle's Medium (DMEM) were incubated at 37°C under 5% CO₂ in a 96 well plate (100 μ L each). After 24 hours, the cells were exposed to graded concentrations of tested compounds and then were placed in an incubator at 37°C.

After 48 hours, the cells were treated with 100 μ L of 0.02% MTT solution (incubated for 3 hours at 37°C). After removal of the solution, residual formazan was dissolved in 100 μ L of DMSO. The absorbance was measured on a microplate reader at 575 nm and the IC₅₀ value was calculated by using a software Kaleidagraph.

Results and Discussion

In this study, we report the isolation of peroxyketal metabolites from a sponge *Plakortis* sp. collected off Irabu Island, Okinawa²⁰ including their activity. Marine sponges belonging to the family Plakinidae have been known as a vast source of natural products particularly for their peroxide and peroxide-derived compounds^{4,5}.

Compound 1a was isolated as a colorless oil (2.4 mg). It showed a pseudo-molecular ion at m/z 363.21429 [M+Na]⁺ in the positive HRESIMS corresponding to the molecular formula C₁₉H₃₂O₅ with four degrees of unsaturation. The ¹H NMR spectrum of compound 1a showed a methyl triplet (δ 0.98, J = 7.5 Hz), a methyl doublet (δ 1.41, J = 7.0 Hz) and a methyl singlet on a double bond (δ 1.71). Of two methoxy singlets (δ 3.27 and 3.72), the latter one is suspected to be from a carbomethoxy group. Three olefinic protons appeared as a triplet (δ 5.38, J = 7.0 Hz) and a doublet (δ 6.06, J = 15.5 Hz) and a multiplet (δ 5.51).

In the ¹³C NMR spectrum, two signals at δ 80.5 and 104.6 may suggest the presence of an endoperoxide group. Based on the HRESIMS and NMR data, compound 1a was determined to be the antifungal compound peroxyplakoric acid B₁ methyl ester¹² (Figure 1).

Compound 2b (1.4 mg) was obtained along with compound 1b (0.5 mg). Both compounds showed similarities in their ¹H NMR spectra to that of compound 1a except the absence of a carbomethoxy signal. HRESIMS data of the pseudo-molecular ion for compound 2b at m/z 349.20188 [M+Na]⁺ indicated the loss of 14 mass units from compound 1a suggesting the presence of a carboxylic acid instead of a methyl ester. The mass data of compound 1b at m/z 349.19687 [M+Na]⁺ indicated that this compound also has a carboxylic acid moiety. Comparison of the NMR data of 2b with those of 1a and 1b, the difference is expected at C-2

and/or C-3 positions due to the chemical shifts of H-2 (δ 2.56), H-2Me (δ 1.26) and H-3 (δ 4.26).

NMR data of compound 2b was similar to that of a known compound peroxyplakoric acid A₁ methyl ester (2a)⁴. Furthermore, methylation of 2b with TMS diazomethane yielded 2a which showed a carboxyl methyl at δ 3.70 on the ¹H NMR spectrum and a mass peak at m/z 363.21366 [M+Na]⁺. This treatment confirmed that the free acid compound 2b is naturally occurring and it is possible to turn 2b into the methyl ester compound 2a during the isolation process. The comparison of ¹H and ¹³C NMR data for the compounds was tabulated as shown in tables 1 and 2.

Compound 1a showed cytotoxicity against NBT-T2 cells with IC₅₀ 8.7 μ g/mL. Compounds 1b, 2a and 2b did not show activity in the cytotoxicity test due to the decomposition process during the assay as it was confirmed by the NMR measurements displaying a mess spectrum totally. Although the cyclic peroxide group was reported to be stable, there might be a condition that leads to a degradation process. The presence of methoxy group in structure 1a is essential for the bioactivity. Furthermore, the stereochemistry in C-2 has a great effect in the cytotoxicity. Schirmeister et al¹⁶ reported the effect of stereochemistry related to presence or absence of the methyl group on the cytotoxicity of endoperoxide. Plakortides, the peroxy compounds isolated from the sponge *Plakortis angulospiculatus* were reported active against some cancer cell line¹⁵.

Compound 3 was isolated as an oil. The molecular formula C₁₁H₁₈O₂ was deduced by negative mode HRESIMS of m/z 181.12285 [M+H]⁺ and ¹³C NMR which required three degrees of unsaturation. Three olefinic protons at δ 6.08 (d, J = 15.5), 5.50 (m) and 5.38 (brt, J = 7.0), a methyl singlet at δ 1.71 and a methyl triplet at δ 0.98 (J = 7.5 Hz) in the ¹H NMR spectrum of 3 (Figure 5) represented typical signals to those of cyclic peroxides side chains as shown in Manadoperoxide¹³.

The lack of seven carbons signals in the ¹³C NMR data, when compared to the peroxyplakoric acids, indicated the absence of a cyclic peroxide along with the methoxy and secondary methyl portions. In addition, a carboxylic acid signal at δ 177.7 in the ¹³C NMR spectrum exhibited the presence of carboxylic acid functionality in the structure. The use of 1D and 2D (COSY and HMBC) NMR including decoupling observations allowed for the conclusion that compound 3 is a new aliphatic unsaturated fatty acid structure as shown in figure 2.

Compound 3 could be a degradation product of cyclic peroxide compounds. The cyclic peroxide moiety might be decomposed through a reduction process to form a diol moiety and give a hemiketal moiety¹⁴. The hemiketal could then turn into a ketone functionality and become an ester through Baeyer-Villiger oxidation¹⁶.

15

Table 1

¹H NMR data for compounds 1a, 1b, 2a and 2b (500 MHz, CDCl₃, J in Hz)

Position	1a	2b	1b	2a
2	3.06 dq (9.5, 7.0)	2.56 m	3.07 dq (7.0, 7.0)	2.51 dq (7.5, 7.5)
3	4.20 dt (9.5, 6)	4.24 m	4.20 ddd (10, 6.0, 6.0)	4.19 m
4	1.43 m	1.50 m	1.42 m	1.52 m
5	1.68 m	1.91 m	1.67 m	1.90 m
6	1.63 m	1.62 m	1.63 m	1.63 m, 1.68 m
7	1.42 m	1.43 m	1.42 m	1.35 m, 1.45 m
8	2.10 m	2.10 m	2.10 m	2.10 m
10	5.51 m	5.51 m	5.51 m	5.51 m
11	6.06 (15.5)	6.05 d (15.5)	6.06 d (15.5)	6.05 d (15.5)
13	5.38 t (7.0)	5.37 t (7.0)	5.38 t (7.0)	5.37 t (7.0)
14	2.15 m	2.11 m	2.15 m	2.15 m
15	0.98 t (7.5)	0.98 t (7.5)	0.98 t (7.5)	0.98 t (7.5)
16	1.14 d (7.0)	1.26 d (7.0)	1.14 d (7.0)	1.25 d (7.0)
17	1.71 s	1.71 s	1.71 s	1.71 s
OCH ₃	3.72 s	-	-	3.70 s
OCH ₃	3.27	3.26 s	3.27 s	3.26 s

Table 2

¹³C NMR data for compounds 1a, 1b and 2b (500 MHz, CDCl₃)

Position	1a	1b	2b
1	175.1	n.o.	179.5
2	41.7	43.0	41.4
3	80.7	80.7	80.5
4	20.4	23.0	20.3
5	26.6	30.4	26.5
6	104.6	102.9	104.6
7	31.0	32.1	31.1
8	22.9	22.9	22.9
9	32.8	32.8	32.8
10	126.3	126.3	126.2
11	135.7	135.6	135.7
12	132.8	132.7	132.8
13	132.8	132.8	132.8
14	21.4	21.4	21.4
15	14.1	14.1	14.1
16	13.8	12.8	13.7
17	12.3	12.3	12.3
1'	51.9	-	-
6'	48.9	48.5	48.9

n.o: not observed

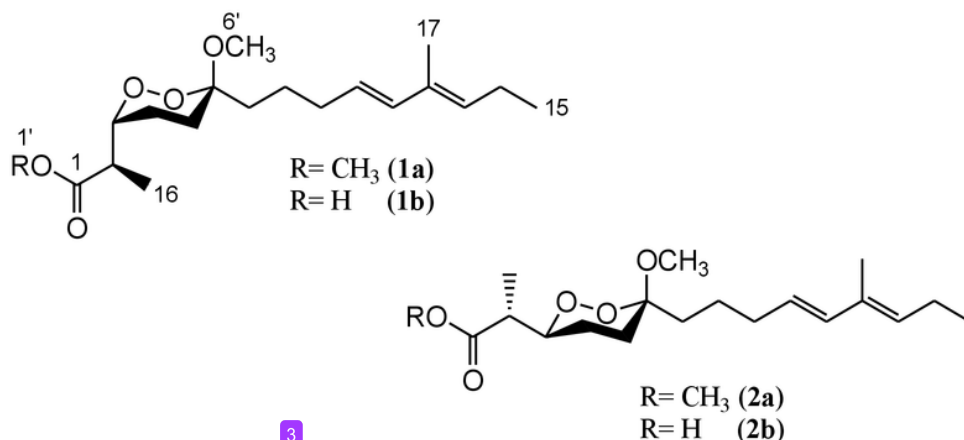


Figure 1: The compounds isolated from the sponge *Plakortis* sp. collected from Okinawa water.

To confirm this hypothesis, follow up experiments would need to be done. The ester was then hydrolyzed to an acid group (Scheme 1). Compound 3 was not active in the cytotoxicity test toward NBT-T2 cells. Our previous research showed, some compounds from marine sponges showed activity against the NBT-T2 cells¹⁷. Other study exhibited that halioxepine inhibited the cell line at 4.8 $\mu\text{g/mL}$ ¹⁷ and other compounds, biaketide and debromo-antiazirine have IC_{50} 8.3 and 4.7 $\mu\text{g/mL}$ respectively.¹⁸

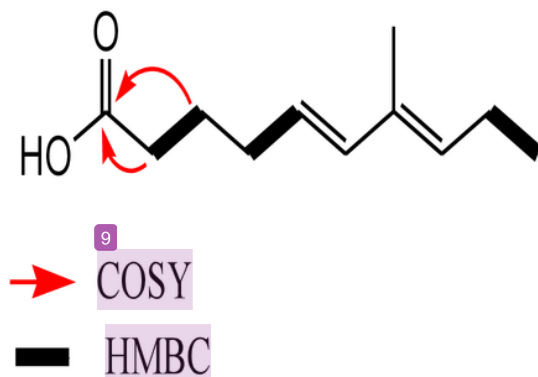
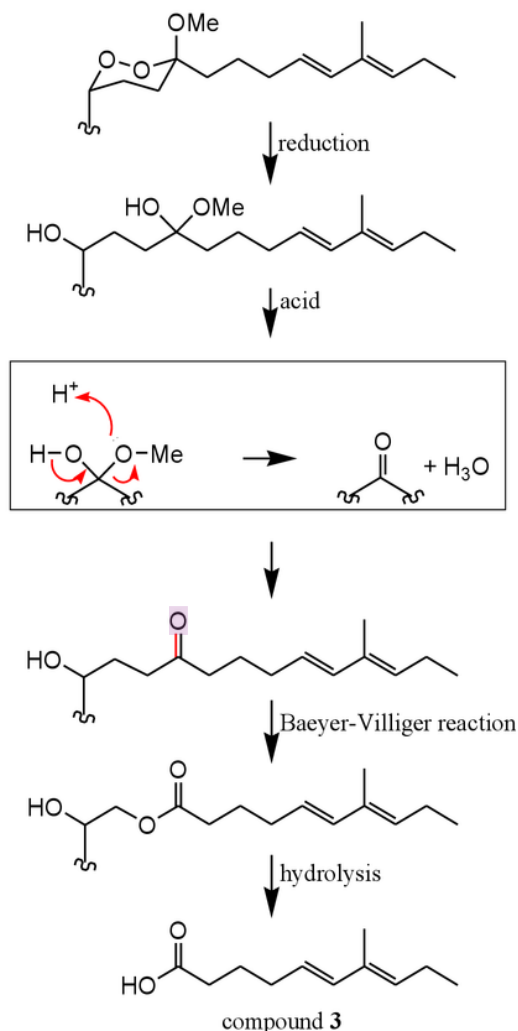


Figure 2: Key COSY and HMBC correlations of compound 3

Conclusion

The investigation of bioactive compounds derived from an Okinawan sponge *Plakortis* sp. revealed cyclic peroxides methyl ester (1a), two new cyclic peroxide acids (1b and 2b) and a fatty acid (3). Compound 1a was active in the cytotoxicity test with IC_{50} 8.7 $\mu\text{g/mL}$ against NBT-T2 cells.

Compound 3 may arise from a base-promoted rearrangement of cyclic peroxide containing the compound. This could occur from a reduction process led by the ring-opening and the acid being formed via Baeyer-Villiger oxidation. This compound has no cytotoxic property which may be due to the lack of cyclic peroxide functional groups.



Scheme 3: Plausible degradation mechanism of peroxyplakoric acids into compound 3

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